A 3-DAY *IN VITRO* STORAGE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) UNFERTILISED EGGS IN COELOMIC FLUID AT 12°C DOES NOT AFFECT DEVELOPMENTAL SUCCESS

by

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ABSTRACT. - Unfertilised rainbow trout (*Oncorhynchus mykiss*) eggs were stored *in vitro* in coelomic fluid (CF) and in trout artificial medium (TAM) for 3 and 9 days at 12°C. Eggs held *in vivo* in the body cavity for 3 and 9 days were obtained from the same females and used as a control. Eggs were fertilised with a pool of sperm obtained from 5 males. The percentage of embryos reaching eyed stage, hatching and yolk-sac resorption, as well as morphological abnormalities were recorded as an index of egg quality (fertility and developmental success). Unfertilised eggs stored 3 days *in vitro* in CF exhibited the same quality as eggs stored 3 days *in vivo* (control). After a 9-day storage in CF, egg quality was lower for the 2 females analysed. For eggs held 3 days in TAM, eying, hatching and swim-up rates were significantly lower than *in vivo*. After 9 days in TAM, fertility and developmental success were extremely low. Unfertilised eggs stored 3 days *in vitro* in CF and *in vivo* (control) exhibited the same percentage of morphological abnormalities at yolk-sac resorption. Surprisingly, no malformation was observed at yolk-sac resorption when unfertilised eggs were stored 3 days *in vitro* in TAM. This study demonstrates that cœlomic fluid can be used at 12°C for external storage of trout eggs for at least 3 days without loss of egg quality. In contrast, a 3-day storage at 12°C in trout artificial medium induces a significant loss of egg quality.

RÉSUMÉ. - Le stockage d'œufs non fécondés de truite (*Oncorhynchus mykiss*) pendant 3 jours dans du liquide cœlomique à 12°C n'affecte pas le développement.

Des ovocytes ovulés de truite arc-en-ciel (*Oncorhynchus mykiss*) ont été prélevés et stockés *in vitro* dans du liquide cœlomique ou dans du milieu artificiel, à 12°C, pendant 3 et 9 jours. Le reste de la ponte a été maintenu dans la cavité abdominale des femelles pendant le même temps, à la même température, et a servi de témoin. Après 3 et 9 jours, les ovocytes issus des 3 situations (*in vivo*, *in vitro* dans du liquide cœlomique et *in vitro* dans du milieu artificiel) ont été fécondés avec un pool de sperme prélevé sur 5 mâles. Le succès du développement a été estimé non seulement à partir du pourcentage d'embryons atteignant le stade œillé, l'éclosion et la résorption de la vésicule vitelline, mais également d'après la proportion d'alevins présentant des anomalies morphologiques à résorption. Des ovocytes non fécondés stockés *in vitro* dans du liquide cœlomique pendant 3 jours présentent les mêmes capacités de développement que le témoin *in vivo*. En revanche, après 9 jours de stockage *in vitro* dans du liquide cœlomique, les capacités de développement observées ont été très faibles pour les 2 femelles analysées. Les ovocytes non fécondés stockés *in vitro* dans du milieu artificiel ont montré des capacités de développement inférieures au témoin *in vivo* après seulement 3 jours de stockage et très faibles après 9 jours de stockage. Les alevins issus d'ovocytes non fécondés stockés 3 jours *in vitro* dans du liquide cœlomique ont présenté la même proportion d'anomalies morphologiques que les ovocytes témoins conservés *in vivo*. En revanche, les alevins issus d'ovocytes conservés 3 jours dans du milieu artificiel n'ont présenté aucune malformation. Cette étude montre que le liquide cœlomique peut être utilisé pour stocker *in vitro* des ovocytes non fécondés, pendant au moins 3 jours, à 12°C alors que le milieu artificiel utilisé dans les mêmes conditions ne permet pas de maintenir de façon satisfaisante la qualité des ovules.

Key words. - Salmonidae - *Oncorhynchus mykiss* - Rainbow trout - *In vitro* storage - Artificial medium - Coelomic fluid - Egg quality - Developmental success.

In fish, ovulated eggs undergo a rather significant and fast decrease of their ability to be fertilised (Bromage *et al*., 1994). While most fish spawn their eggs soon after ovulation, salmonids can hold their eggs in the abdominal cavity for at least a week without loss of fertility (Springate *et al*., 1984). However, holding success can be affected by high temperatures. During this period, eggs are bathed in a fluid known as "coelomic" or "ovarian" fluid.

In contrast, *in vitro* storage of ovulated eggs in coelomic fluid results in a rapid decrease of egg fertility and is even more dependent on holding temperature. For example, fertility could be maintained for several days at low temperature (below 4°C) while it significantly decreased at 12-13°C after a few hours (Withler and Morley, 1968; Billard *et al*., 1981).

However, it was recently shown that rainbow trout

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(*Oncorhynchus mykiss*) unfertilised eggs could be held at 12-13°C in artificial medium for 48 h without loss of fertility (Goetz and Coffman, 2000).

The purpose of the present study was to investigate medium (3 days) and long (9 days) term *in vitro* storage at 12°C of rainbow trout ovulated eggs in coelomic fluid and artificial medium. While most studies focused on egg fertility, the present study also investigates the effects of *in vitro* storage on early development (up to yolk-sac resorption) and includes survival rates as well as the occurrence of morphological abnormalities.

MATERIALS AND METHODS

Fish

Two-year old male (1.2-1.7 kg) and female (1.3-1.8 kg) rainbow trout (*Oncorhynchus mykiss*), from a spring-spawning strain, were obtained from INRA experimental farm (Sizun, France) and held in a recirculated water system at 12°C, under natural photoperiod in INRA experimental facilities (Rennes, France).

Egg and milt collection

For gamete collection, fish were anaesthetised in 0.05% 2-phenoxy-ethanol. Females were checked for ovulation three times a week. On day 0, approximately 600 eggs were obtained from ovulated females (24 h-48 h after ovulation) by applying pressure to the abdomen. Eggs (~150) were collected from the same females on day 3 and day 9 to obtain controls held *in vivo*. To collect milt, the genital region was wiped and milt was obtained by manual stripping. Motility was evaluated as previously described (Labbe and Maisse, 2001). A pool was made from 5 males exhibiting a motility above 90% and stored on ice until fertilisation.

Incubation media

A pool of coelomic fluid (CF) was made from 6 ovulated females (2-8 days after ovulation). Females were stripped and the coelomic fluid was isolated by pouring the eggs onto a screen suspended over a beaker. CF was then centrifuged (10 mn, 4,000 g, 4°C) coelomic fluid was used to hold eggs from 3 females while unfiltered coelomic fluid was used to hold eggs from 3 other females.

Filtered (0.22 µm) trout artificial medium (TAM; 132 mM NaCl, 3.1 mM KCl, 1.0 mM $MgSO_4$, 3.4 mM $CaCl_2$, 5.6 mM glucose, 20 mM Hepes, pH 8.0 et 305 mOsm) was used to hold eggs *in vitro*.

Storing eggs

In vitro storage was performed, under sterile conditions, using 6-well polystyrene culture plates (Costar, USA). Aliquots of 30 eggs were distributed in separate wells containing 3 ml of storage medium. For each female, 4 replicates of 30 eggs were used for each incubation condition. Before storage in artificial medium, eggs were washed three times with 3 ml of TAM to remove residual coelomic fluid. All plates were kept in the dark, in a 12°C incubator, under gentle and constant agitation (50 rpm).

This experiment was repeated twice on three different females.

Fertilisation

Fertilisation was performed as previously described with minor modifications (Billard and Jalabert, 1974). On day 3 and day 9, eggs (120-150) were transferred into plastic tubes (50 ml) and washed with 5 ml of Ovafish® (IMV, l'Aigle, France). Milt was added (5 µl) along with 5 ml of Actifish® (IMV, l'Aigle, France), tubes were gently swirled and incubated for 10 minutes.

After discarding Actifish, eggs were transferred into plastic incubators and held in the dark at 10°C in a recirculated water system during all early development (55 days).

Developmental success monitoring

Incubators were checked every day to remove unfertilised eggs or dead embryos. The number of embryos reaching eyed-stage, hatching and swim-up was carefully monitored. At yolk-sac resorption, fish were over-anaesthetised in 2-phenoxy-ethanol and morphological abnormalities were counted on digital pictures to calculate the percentage of fry exhibiting obvious malformations. Seven types of morphological abnormalities were identified: incomplete yolk-sac resorption, cyclop, prognate, siamese, spinal cord torsion, spiraloid torsion and absence of caudal fin. Each individual alevin was carefully checked for each type of malformation.

Statistical analysis

Data was converted by the arcsine transformation and statistical analysis was performed using t tests for paired observations.

RESULTS

Results from a female exhibiting a very low swim-up rate for eggs held 3 days *in vivo* and no survival for eggs held 9 days *in vivo* were not included in the statistical analysis.

Storage in coelomic fluid

Eggs held 3 days *in vitro* in CF exhibited eying, hatching

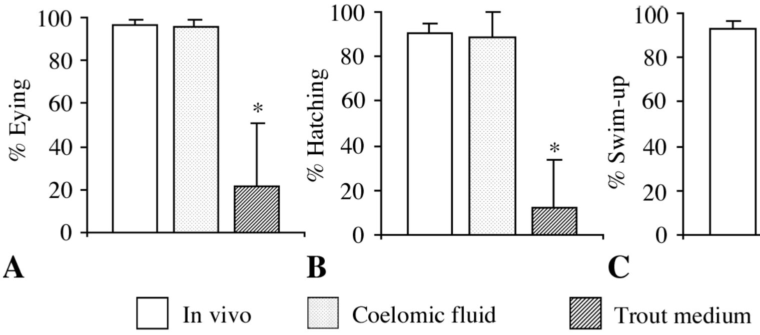

Figure 1. - Effects on eying (**A**), hatching (**B**) and swim-up (**C**) rates of a 3-day *in vitro* storage of unfertilised rainbow trout eggs in coelomic fluid or trout artificial medium and *in vivo* ageing in the body cavity (control). *: Significantly different from control ($p < 0.01$). All bars represent the means ± standard deviation of experiments conducted on the eggs of 5 females.

and swim-up rates that were not significantly different from eggs held *in vivo* (Fig. 1A-C). The percentage of malformations observed in eggs held three days *in vitro* in CF was not significantly different from the control (Fig. 2).

On day 9, eggs from 3 females that had been stored *in vitro* in unfiltered CF were contaminated by bacterial agents and could not be fertilised. Eggs from 2 other females, held 9 days in filtered coelomic fluid (0.2 µm), could be fertilised and the developmental success observed *in vitro* was lower than *in vivo* (Fig. 3, Tab. I). However, this could not be statistically tested.

Storage in trout artificial medium

Eggs held 3 days *in vitro* in TAM exhibited eying, hatching and swim-up rates that were significantly lower than *in vivo* (Fig. 1A-C). In addition, the developmental success observed in TAM after a 3-day storage was highly variable among females. Surprisingly, no morphological abnormality was detected at yolk-sac resorption (Fig. 3).

Eggs held 9 days *in vitro* in TAM exhibited eying, hatching and swim-up rates that were significantly lower than *in vivo* (Fig. 3A-C). As a matter of fact, only a few individuals reached yolk-sac resorption.

DISCUSSION

Previous studies reported that external storage of salmo-

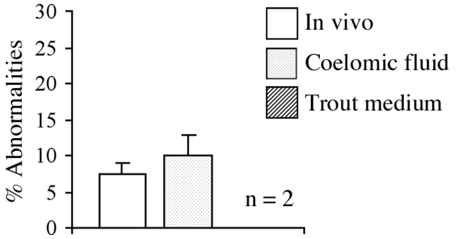

Figure 2. - Effects of a 3-day *in vitro* storage of unfertilised rainbow trout eggs in coelomic fluid or trout artificial medium and *in vivo* ageing in the body cavity (control) on morphological abnormalities observed at yolk-sac resorption. *In vivo* and coelomic fluid bars represent the means ± standard deviation of experiments conducted on the eggs of 5 females. For trout artificial medium, means were calculated from 2 females.

Table I. - Eying, hatching and swim-up rates of rainbow trout eggs after a 9-day *in vitro* storage in filtered coelomic fluid (CF) and trout artificial medium (TAM) and *in vivo* storage in the body cavity (control).

	Female	*In vivo* control	*In vitro* CF	*In vitro* TAM
% Eying	1	98.9	67.5	0.0
	2	95.5	45.8	0.0
% Hatching	1	98.9	64.2	0.0
	2	74.4	11.7	0.0
% Swim-up	1	95.0	60.9	0.0
	2	68.8	9.2	0.0

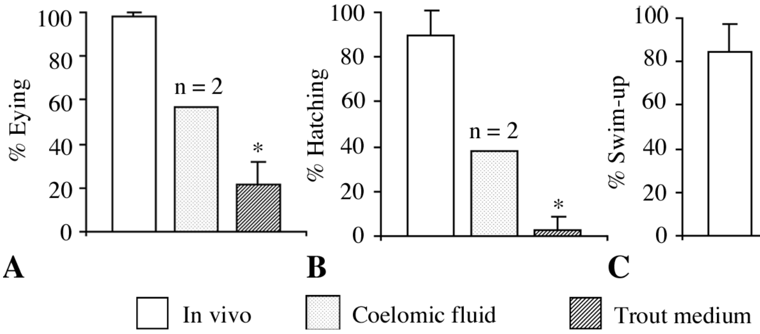

Figure 3. - Effects on eying (**A**), hatching (**B**) and swim-up (**C**) rates of a 9-day *in vitro* storage of unfertilised rainbow trout eggs in coelomic fluid or trout artificial medium and *in vivo* ageing in the body cavity (control). *: Significantly different from control ($p < 0.01$). *In vivo* and trout medium bars represent the means ± standard deviation of experiments conducted on the eggs of 5 females. For coelomic fluid means were calculated from 2 females.

nid eggs in coelomic fluid (CF) was highly dependent on duration and holding temperature. For low temperatures, storage is possible for several days while higher temperatures can only be used for short-term storage. At 4°C for example, trout (*Salmo trutta fario*) eggs stored for 72 h exhibited a fertility of 61- to 90% (Billard *et al*., 1981). In contrast, *in vitro* storage in CF has always resulted in a rather rapid loss of fertility for temperatures close to 12°C. In sockeye salmon and pink salmon, a fertility around 35% was observed after a 72-h *in vitro* storage in CF at 8-9°C (Withler and Morley, 1968). In addition, chum salmon egg fertility could be maintained *in vitro* in CF for 40 h at 12°C, but decreased to 28% for a 82-h storage (Jensen and Alderdice, 1984). Furthermore, a study demonstrated that after a short-term (28-76 h) storage at 10 and 15°C, trout (*Salmo trutta*) egg fertility significantly decreased (Billard and Gillet, 1981). In addition, rainbow trout eggs obtained after spawning induction exhibited a complete loss of fertility after a 24-h storage at 19°C or a 52-h storage at 10°C (Billard and Gillet, 1981). In contrast, a recent study showed that a 2-day *in vitro* storage in CF at 12°C does not induce a significant loss of rainbow trout egg fertility (Goetz and Coffman, 2000). In the present study we observed a maintain of trout egg fertility after a 3-day *in vitro* storage in CF at 12°C. Thus, our observations are in agreement with prior study performed on naturally ovulating rainbow trout (Goetz and Coffman, 2000). Differences in fertility observed among experiments could be partly explained by the holding time in the body cavity before *in vitro* storage, especially in experiments performed on salmons captured from the wild (Jensen and Alderdice, 1984). Furthermore, we observed that eggs stored 3 days *in vitro* in CF were able to develop normally throughout early development. Thus it is possible to store rainbow trout eggs at 12°C in CF for at least 3 days without loss of fertility nor lowering of developmental success. In addition, we observed a fertility around 50% and a normal early development in eggs stored 9 days at 12°C in filtered coelomic fluid. This observation suggests that *in vitro* storage in CF at 12°C can be performed for more than 3 days without affecting drastically egg quality.

In vitro storage of unfertilised eggs in artificial media was mostly studied for short-term periods. For example, short-term storage (5-10 min) of unfertilised rainbow trout eggs in saline solution (0.9% NaCl, 312 mOsm) and fish Ringer solution is possible without loss of fertility (Erdahl *et al*., 1987). Storage up to 30 minutes in artificial media did not decrease the fertility of coho salmon eggs (Wilcox *et al*., 1984), and short-term (up to 40 min) storage of trout eggs was also reported in saline solutions containing carbonate-bicarbonate buffers (Billard and Jalabert, 1974). Rainbow trout eggs stored in several artificial media (278-308 mOsm) experienced a decrease in fertility after 40 minutes (Billard and Jalabert, 1974). All those studies concluded to a rapid loss of egg quality after an *in vitro* storage in artificial medium. However, a recent study showed that an artificial medium (Cortland medium; 124.1 mM NaCl, 5.1 mM KCl, 1.0 mM $MgSO_4$, 1.6 mM $CaCl_2$, 5.6 mM glucose, 20 mM Hepes), could be used to store rainbow trout eggs for at least 2 days at relatively high temperatures (12-13°C) without loss of fertility (Goetz and Coffman, 2000). In the present study, we observed a significant loss of egg quality (fertility and developmental success) after a 3-day *in vitro* storage in trout artificial medium (TAM). Thus, it is possible that trout egg fertility can not be maintained in artificial medium for more than 2 days at 12°C. However it is also possible that TAM composition is responsible for the loss of egg fertility observed after a 3-day *in vitro* storage. While TAM is chemically close to Cortland medium, its pH is lower (8.0 *vs* 8.5) and its osmolality is higher (305 *vs* 290). In addition, Cortland medium contains antibiotics (penicillin 100 IU/ml and streptomycin sulphate 0.1 mg/ml) while no antibiotic was used in the present study. Finally, TAM evidently lacks some CF components that could play a key-role in maintaining egg fertility *in vitro* at 12°C for more than 3 days. For example, it was suggested that trout ovulatory proteins (TOPSs), secreted in the coelomic fluid after ovulation, could play a role in the protection of ovulated brook trout (*Salvelinus fontinalis*) eggs against bacterial infections (Coffman *et al*., 2000).

Concerning morphological abnormalities, no difference was observed between *in vivo* and *in vitro* storage in coelomic fluid. This demonstrates that a 3-day *in vitro* storage in CF at 12°C allows normal embryonic development. Interestingly enough, no abnormality was observed after *in vitro* storage in TAM. Therefore it is possible that TAM only allows normal embryos to develop. This would suggest that mechanisms involved in the occurrence of abnormalities take place during post-ovulatory egg ageing.

CONCLUSION

The present study demonstrates that unfertilised rainbow trout eggs can be maintained for at least 3 days *in vitro* in coelomic fluid, at 12°C, without loss of quality (fertility and developmental capacity). In addition, our observations suggest that a 9-day storage in filtered coelomic fluid does not totally alter egg fertility and developmental capacity. In contrast, a 3-day *in vitro* storage in trout artificial medium induces a significant loss of egg fertility and a very low survival at yolk-sac resorption.

REFERENCES

BILLARD R. & B. JALABERT, 1974. - L'insémination artificielle de la truite (*Salmo gairdneri* Richardson). *Ann. Biol. Anim. Bioch. Biophys.,* 14: 601-610.

BILLARD R. & C. GILLET, 1981. - Ageing of eggs and temperature potentialization of micropolluant effects of the aquatic medium on trout gametes. *Cah. Lab. Montereau*, 12: 35-42.

BILLARD R., MARCEL J. & D. MATEI, 1981. - Survie *in vitro* et *post mortem* des gamètes de truite fario (*Salmo trutta fario*). *Can. J. Zool.*, 59: 29-33.

BROMAGE N., BRUCE M., BASAVARAJA N., RANA K., SHIELDS R., YOUNG C., DYE J., SMITH P., GILLESPIE M. & J. GAMBLE, 1994. - Egg quality determinants in finfish: The role of overripening with special reference to the timing of stripping in the Atlantic halibut *Hippoglossus hippoglossus*. *J. World Aquacult. Soc.*, 25: 13-21.

COFFMAN M.A., PINTER J.H. & F.W. GOETZ, 2000. - Trout ovulatory proteins: site of synthesis, regulation, and possible biological function. *Biol. Reprod.*, 62: 928-38.

ERDAHL A.W., CLOUD J.G. & E.F. GRAHAM, 1987. - Fertility of rainbow trout (*Salmo gairdneri*) gametes: Gamete viability in artificial media. *Aquaculture*, 60: 323-332.

GOETZ F.W. & M.A. COFFMAN, 2000. - Storage of unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) in artificial media. *Aquaculture*, 184: 267-276.

JENSEN J.O. & D.F. ALDERDICE, 1984. - Effect of temperature on short-term storage of eggs and sperm of chum salmon. *Aquacult*ure, 37: 251-265.

LABBE C. & G. MAISSE, 2001. - Characteristics and freezing tolerance of brown trout spermatozoa according to rearing water salinity. *Aquaculture*, 201: 287-299.

SPRINGATE J.R.C., BROMAGE N.R., ELLIOTT J.A.K. & D.L. HUDSON, 1984. - The timing of ovulation and stripping and their effects on the rates of fertilisation and survival to eying, hatch and swim-up in the rainbow trout (*Salmo gairdneri* R.). *Aquaculture*, 43: 313-322.

WILCOX K.W., STOSS J. & E.M. DONALDSON, 1984. - Broken eggs as a cause of infertility of coho salmon gametes. *Aquaculture*, 40: 77-87.

WITHLER F.C. & R.B. MORLEY, 1968. - Effects of chilled storage on viability of stored ova and sperm of sockeye and pink salmon. *J. Fish. Res. Board Can.*, 25: 2695-2699.

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